

**ANTI-CANCER ACTIVITY OF GREEN SPORAL POLYKETIDE
FROM *STREPTOMYCES SP-JB87* ISOLATED FROM *JASMONIUM
BREVILOBUM***

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ABSTRACT

Actinomycetes were isolated from rhizosphere soil of medicinal plants from Dodabetta forest and 8 strains were selected based on unique aerial spore pigment and morphology. Aerial spores were harvested from actinomycetes grown on solid state media. DMSO-Ethylacetate extraction and precipitation of Sporal polyketide pigments were made and screened for its *in vitro* anti-cancer activity against A549 cell lines. The selected green sporal polyketide (GSP) pigment was purified by Fractional precipitation and two distinct fractions were observed in HPLC-PDA detector system. FTIR and UV-Visible spectral analysis revealed the presence of protomelanin like polyketide precursors responsible for melanin formation in aerial spore walls. The GSP extracted from *Streptomyces sp.* JB87 isolated from *Jasmonium*

brevilobum showed EC₅₀ value of 70 µg/ml against A549 lung cancer cell lines.

KEYWORDS: A549 cell lines; Green Sporal Polyketide (GSP); FTIR; HPLC; Protomelanin; *Streptomyces sp-JB87*.

1. INTRODUCTION

Rhizosphere actinomycetes is of particular interest as they participate in the protection of the host plant from root infection. According to direct microscopic studies, actinomycete mycelium constitutes 12 to 20% of the total bacterial biomass in the rhizosphere and exhibit pronounced lytic activity and is able to produce antibiotics and other biologically active compounds.^[1] The content of actinomycete mycelium is significantly higher in the root

system of healthy plants than in the rhizosphere of diseased ones.^[2] They have become increasingly important as producers of secondary metabolites of therapeutic nature. Antagonism is widespread in rhizosphere microflora especially among *Bacillus*, *Pseudomonas*, and *Streptomyces* spp., from which a wide range of biologically active secondary metabolites were isolated.^[3]

The distinct feature of actinobacteria is their ability to exhibit two distinct morphologies i.e vegetative substrate mycelia and asexual aerial spores.^[4] The genus *Streptomyces* is also considered as an interesting model for cell differentiation studies. In its cell cycle this organism is capable of forming spores specialized in survival and reproduction functions. These spores, although not in acryptobiotic state, have several properties in common with true spores and are thus considered as spores in a dormant or resting state. Under adequate stimuli, these spores lose their typical features and undergo germination, resulting in a vegetative mycelium.^[5]

Most of the spore pigmentation in *Streptomyces* spp. Originate from the production of polycyclic aromatic polyketides during the maturation of the spores in the aerial mycelium. Attempts to purify the spore pigment have failed, possibly indicating its covalent attachment to a macromolecular component of the spore.^[6] The polyketide nature of the spore pigment was initially predicted from the sequence of the complex locus (*whiE*) that specifies it. Polyketides makeup the bulk of the therapeutic compounds produced for treating various infections and pathological conditions. It finds application as anti-microbial, anti tumour agent's metal chelators and drug carriers.^[7] The *whiE* genes encode proteins that closely resemble the components of type II polyketide synthases, which are involved in the synthesis of a variety of aromatic antibiotics, including actinorhodin from *Streptomyces coelicolor*, oxytetracycline from *Streptomyces rimosus*, tetracenomycin from *Streptomyces glaucescens* and granaticin from *Streptomyces violaceoruber*.^[8]

Since most of the secondary metabolites are obtained through submerged fermentation through substrate mycelia, the role of the aerial spores is ignored. Sporal polyketides have been generally neglected and under explored due to over emphasis on submerged fermentation methods and difficulty in culturing on a solid substrate and extraction.

The scope of the present study is to isolate and characterize metabolites from distinct aerial spores of actinobacteria isolated from rhizosphere soil of selective medicinal plants and assessing their anticancer properties against cancer cell lines *in vitro*.

2. METHODOLOGY

2.1. MATERIALS

All the chemicals, salts and organic solvents used in the present study were of analytical grade. The growth media required for microbial analysis were procured from Hi Media laboratories, Mumbai, India.

2.2. Isolation and Culture conditions^[9]

The soil samples were air dried aseptically and subjected to 7-8 fold serial dilution and 0.1 ml of each dilution was spread on the Starch Casein Nitrate (SCN) agar and Cellulose Integrated Mineral media, supplemented with 50 µg/ml flucanazole and 1.43 µg/ml Crystal Violet. Strains producing pigmented aerial mycelia were isolated from the rhizosphere soil of the plants *Jasminum brevilobum*, *Commelina clavata*, *Potentilla indica*, *Arisaema tortiosum* and *Simlax aspera* from Western Ghats, India. The isolates were sub-cultured periodically and incubated at 37°C for 7 days to achieve good sporulation and pigmentation which was then preserved in 20% glycerol at -80°C.

2.3. Screening against A549 cell lines

Strains containing pigmented sporal polyketides were isolated based on their sporal pigmentation on petridishes and were harvested after 4 days from the agar surface using a scraper and dried. The spore biomass from each strain about 100mg, was subjected to extraction using DMSO and screened for anticancer activity using MTT assay against A549 lung cancer cell lines.

2.4. Identification and Characterization of select isolates

The strain exhibiting maximum anti-proliferation activity was further identified on the basis of microscopic, physiological and biochemical analysis. The pure cultured isolate was identified to genus level according to Bergey's Manual of Determinative Bacteriology after direct microscopic observation of the vegetative and aerial mycelium at 400x magnification from the cover slip culture,^[10] colour of spore mass and diffusible pigment production were visually estimated using a color chart.^[11]

2.5. Extraction and Purification.

The green sporal polyketide from the selected strain was extracted using DMSO in the ratio 1:10 w/v biomass. The extract was subjected to centrifugation at 4000 rpm for 5 minutes and the supernatant was filtered. The green pigment was partially purified using fractional precipitation by diluting the DMSO extract with Ethyl acetate in 1:2 ratio incubated at -21 °C for 24 hrs.^[12] The resulting precipitate was centrifuged at 10000 rpm for 5 minutes. The pellet obtained was washed with water, methanol and ethyl acetate to remove other polar impurities. The solubility of the resulting polyketide was also tested against various polar solvents. The purity of the compound was tested in HPLC PDA detector system at 400nm with a flow rate of 0.5m/min with the mobile phase Acetonitrile 80% and Methanol/Ethyl acetate (80:20) 20% at room temperature.^[13]

2.6. Compound Characterization

Partial characterization of the polyketide was performed using Spectral scanning in UV Vis spectrophotometer and FTIR.

2.7. MTT Cell Proliferation assay^[14]

The number of viable A549 cells after GSP treatment was evaluated by the MTT (3-[4, 5-methylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay. A549 cells (1×10^4 cells/well) were seeded in a 96-well plate and kept overnight for attachment. After 24 hrs the medium was replaced with fresh medium with various concentrations of GSP and cells were allowed to grow for 24 and 48 h. Four hours before completion of incubation, 10 μ l of MTT (10 mg/ml) was added in each well. After completing the incubation, 100 μ l of solubilization buffer (10% SDS with 0.01 N HCl) was added to each well and incubated overnight at room temperature. Colour developed after the reaction was measured at 550 nm using Epoch - BioTek microplate reader.

2.8. Statistical Analysis

All analyses were performed in triplicate and values expressed as the mean \pm standard deviation. Data analysis was carried out using standard Microsoft Excel data sheet.

3. RESULTS AND DISCUSSION

3.1. Isolation

Among the rhizosphere soil samples collected from the 5 medicinal plant species 101 distinct colonies were isolated in total, based on colony morphology and reverse pigmentation. The

list of strains isolated from each plant species were enumerated in (Table. 1). Khamna et al,^[15] isolated a total of 445 actinomycete from 16 medicinal plant rhizosphere soils, whose morphological and chemotaxonomic studies indicated that 89% of the isolates belonged to the genus *Streptomyces*, only 11% were non-*Streptomyces*.

3.2. Screening for sporal polyketide with anti-proliferation activity

Among the 101 isolates 10 strains exhibiting distinct aerial spore morphology were selected and on screening for the extract producing the maximum anti-proliferation activity against A549 lung cancer cell lines *in vitro*, the extract from the strain JB87 isolated from *Jasmonium breviloenum* exhibited maximum anti-proliferation activity of 84.73% at 50 µl of the crude extract. (Fig. 1)

3.3 Identification of strain.

The screened isolate JB 87 was further identified to be Gram positive bacteria and exhibited filamentous growth in the agar medium and was found to produce chains of aerial spores under microscopic observation (Fig.2a, 2b). Biochemical tests revealed that the strain exhibited biochemical properties predominantly similar to the genus *Streptomyces* which was confirmed on ISP medium 1-7(Fig.2c, 2d) (Table. 2).

Based on the results the strains was identified as *Streptomyces sp.* and was assigned the name *Streptomyces sp. JB 87*.

3.4. Extraction and partial purification of sporal polyketides

The spore biomass extracted from *Streptomyces sp. JB 87* was pale green in colour and the DMSO extract was found to be dark bottle green colour and assigned as Green Sporal Polyketide (GSP). After fractional precipitation using Ethyl acetate at -21 °C the resulting precipitate was obtained as dark greenish powder. The total yield of spore biomass was 2.313g/L and the total compound yield was found to be 63.4 mg/L approx. It was found to primarily insoluble in Methanol, Butanol, Ethyl acetate and Petroleum ether. It was sparingly soluble in water and readily soluble in DMSO and 1M KOH solution. In KOH solution the compound changed colour to a brown solution which might be possibly due to oxidation. The HPLC analysis of the compound revealed the presence of two distinct compound 61.38% and 38.62% at 5.48 and 6.08 retention time respectively (Fig. 3).

It was observed that no pigmented metabolites were produced during the submerged fermentation of the isolate in broth culture. Hence the extracted GSP are exclusively found to be produced in the aerial mycelia in solid state growth phase.

3.5 Spectral Characterization

The spectral characterization of GSP revealed the presence of three distinct peaks at 246nm, 400nm and 695nm in DMSO. On dissolving the compound in 1M KOH solution the peaks at 400nm and 695nm completely disappeared, revealing a gradient spectral graph with no distinct peaks from 200nm to 800nm indicating the formation of melanin as a result of KOH oxidation. There is also a significant increase in the overall absorbance of the pigmented compound throughout the spectrum indicating increase absorption of electromagnetic radiation. The presence of high absorbance below 300nm reveals that the compound has high Ultraviolet radiation absorbance and quenching similar to melanin (Fig.4).

The FTIR spectrum reveals the presence of strong 3409 peak indicating the presence of OH stretch suggesting the presence of phenolic groups. The medium peak at 2925 is within the C-H stretch region of 3000-2850 revealing the presence of alkane group. The strong peak at 1635 indicates the strong presence of primary amine group. Presence of C-C in an aromatic ring was suggested by a peak at 1404. The strong peak at 1247 indicates the presence of C-O with the possibility of carboxylic or ester groups. The medium peak at 1044 indicates the presence of aliphatic NH group (Fig. 5).

3.6. MTT cell proliferation assay

As shown in Fig 6A. GSP inhibited A549 cell proliferation in dose dependent manner. From examination of A549 cells morphology at 24 h after gradient concentration treatment of GSP extract, it was observed that cells started shrinking showing the symptoms of the cell death. Further, total cell viability were measured after treating cells with GSP for 24 h. From Fig. 6B, it was clear that the percent viability of cells started decreasing in the GSP treated group and this effect was observed in a concentration dependent manner. There is significant variation between the control and treated cell as the elongated morphology of the A549 cells have undergone shrinking and the morphology have become circular. The EC50 concentration was derived approximately at 70 µg/ml.

Vijayabharathi *et al.*,^[16] isolates *Streptomyces aurantiacus* AAA5 from humus soil of Western ghats, India, which was found to produce large quantities of resistomycin (52.5 mg/L) and

showed potent cytotoxic activity against HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) cell lines *in vitro*, with EC₅₀ of 0.006 and 0.005 µg/ml, respectively. Che *et al.*,^[17] reported the isolation of a hybrid isoprenoid polyketide named indotertine isolated from reeds rhizosphere soil collected from the mangrove conservation area of Guangdong province, China, and it was found to exhibit significant cytotoxicity against P388 cells. A *Streptomyces* sp. (Lv1-48) obtained from the rhizosphere of the plant *Juniperus excelsa* isolated in Ukraine, produced a linear polyketide, juniperolide.^[18]

List of Tables

Table.1 List of Medicinal plants from which actinobacteria were isolated.

S.no	Plant Species	No of distinct colonies isolated
1.	<i>Arisaema tortiosum</i>	21
2.	<i>Commelina clavata</i> ,	17
3.	<i>Jasminium breviloebum</i> ,	20
4.	<i>Potentilla indica</i> ,	34
5.	<i>Simlax aspera</i>	09

Table. 2: Biochemical tests for *Streptomyces* sp. JB87

S. No	Characteristics	Results
1	Colony morphology	Circular
2	Pigment	Pale Green
3	Gram's reaction	Positive
4	Indole test	Negative
5	Methyl red test	Positive
6	VogesProskauer test	Negative
7	Citrate utilization	Positive
8	Urea hydrolysis	Positive
9	Catalase test	Positive
10	Oxidase test	Positive
11	Nitrate test	Positive
12	Triple Sugar Iron agar	A/A
13	Gelatin hydrolysis	Positive
14	Sucrose	Negative
15	Glucose	Positive
16	Lactose	Positive
17	Mannitol	Positive
18	Galactose	Positive
19	Fructose	Negative
20	Mannose	Positive
21	Starch	Positive
22	Aerial Mycelium	Pale green
23	Substrate mycelium	White

List of Figures

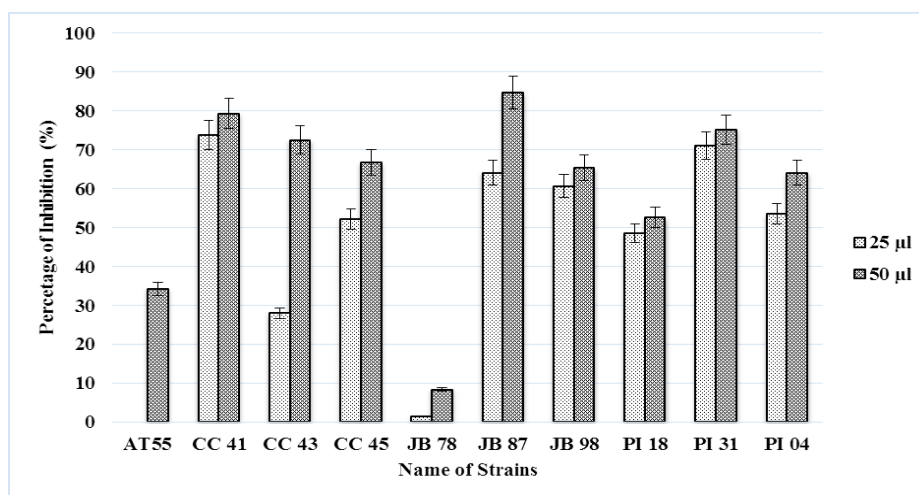


Figure 1. Screening of anti-proliferation activity against A549 cell lines in *in vitro* using extracts from pigmented spore producing strains.

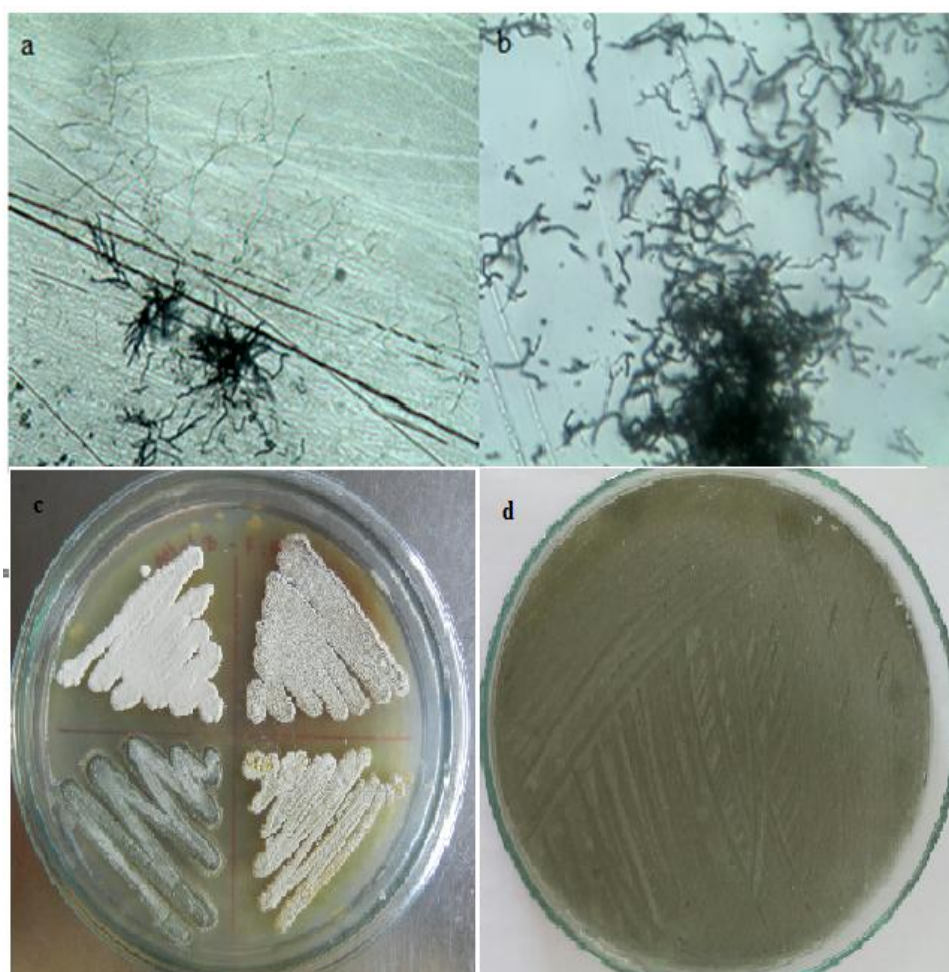


Figure 2. Microscopic observation of *Streptomyces* sp., JB87 at 400x magnification
a: substrate mycelium b: aerial mycelium, c: Colony morphology of isolated strains, d: Pigmentation in aerial mycelia of *Streptomyces* sp. JB87

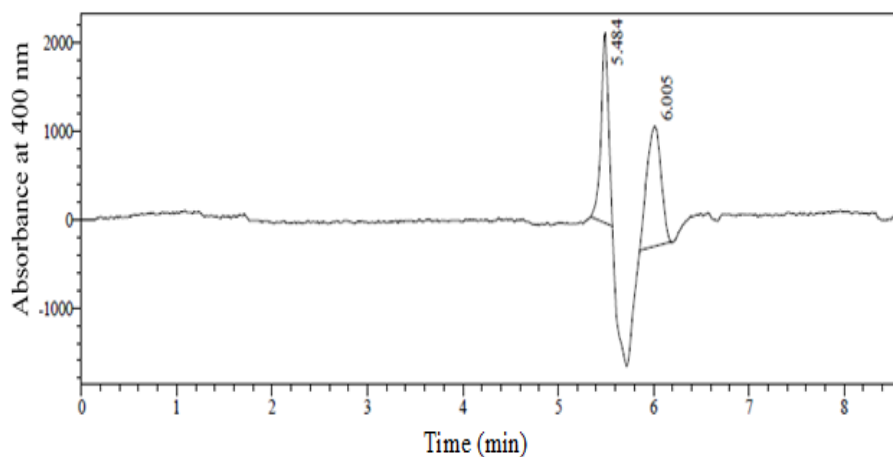


Figure 3. HPLC chromatogram of partially purified GSP

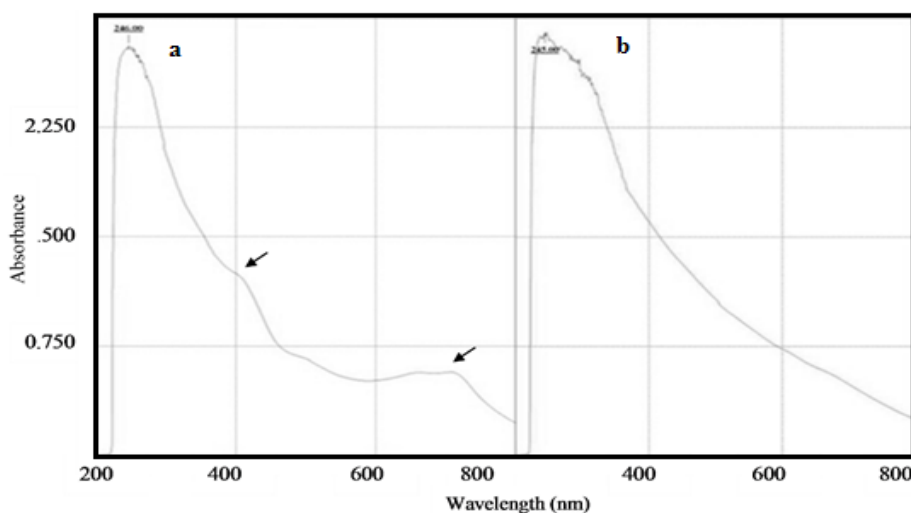


Figure 4. UV Vis spectrophotometric spectrum of GSP. a: Spectral graph of GSP in DMSO showing peaks at 246nm, 400nm and 695nm respectively. b: Spectral graph of GSP in 1M KOH with peak only at 246nm

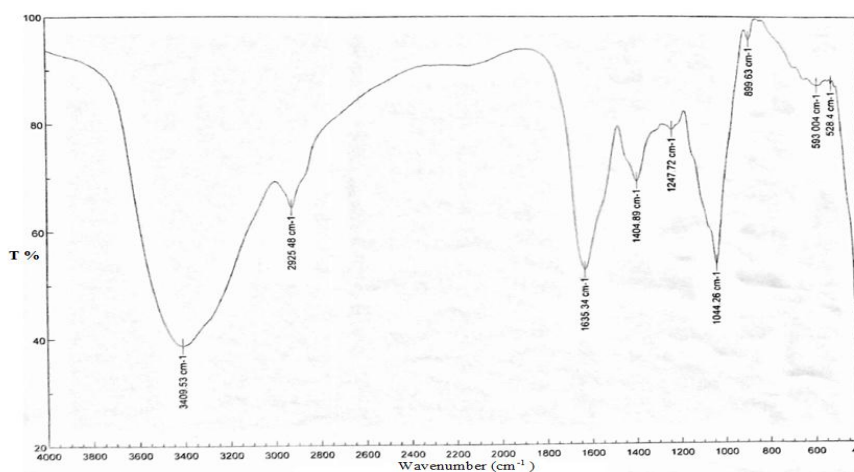


Figure 5. FTIR spectra indicating major functional groups in GSP

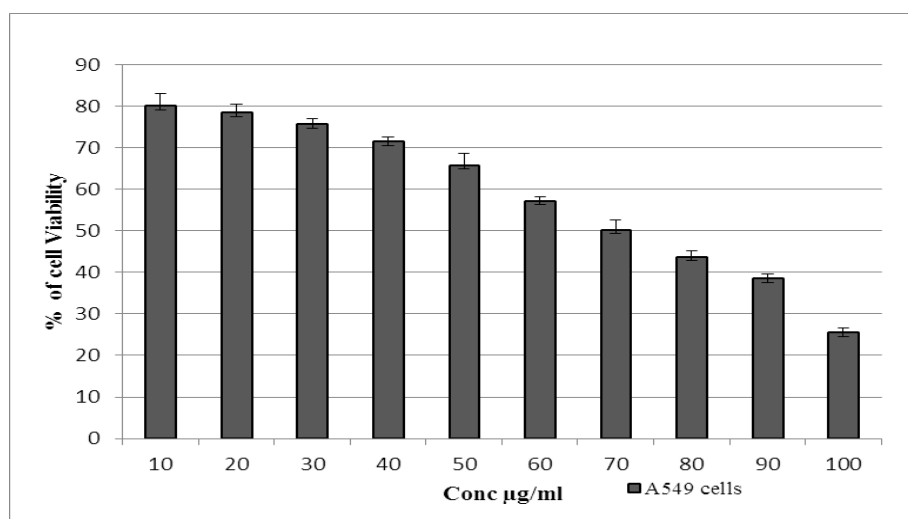


Figure 6A. Inhibition of A549 cell proliferation by GSP. In brief 1×10^4 cells were treated with 0 to 100 µg/ml of GSP.

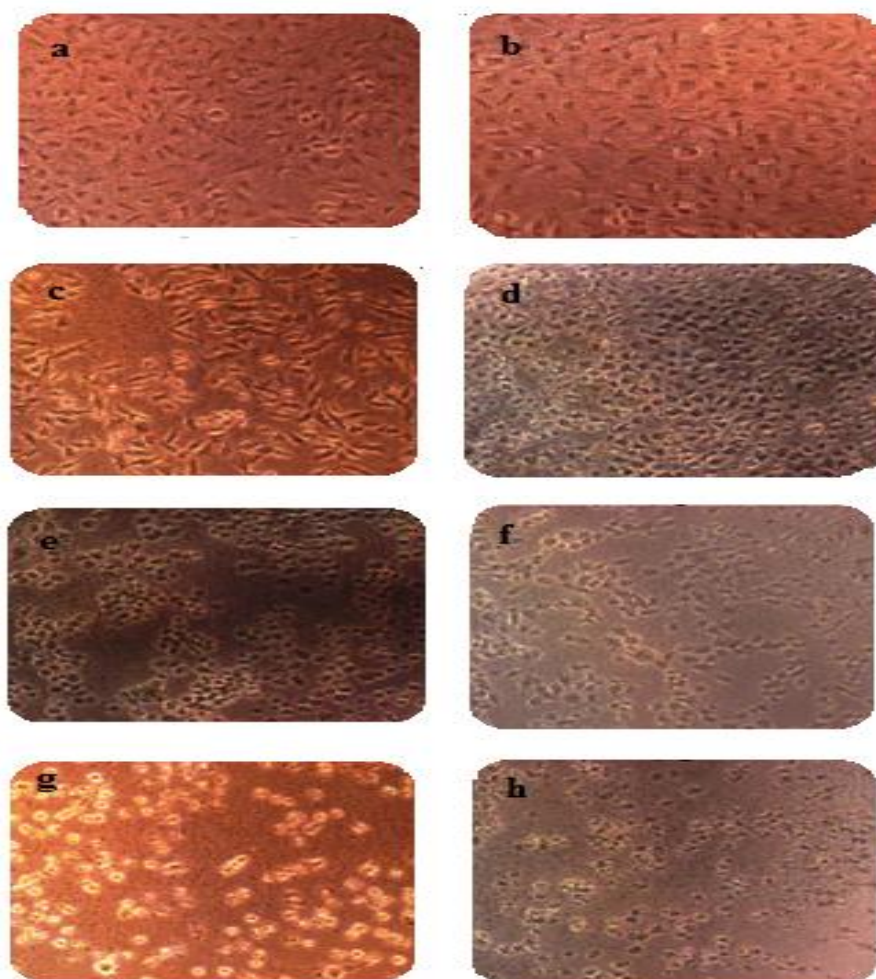


Figure 6B. Morphology of A549 cells treated with different concentrations of GSP. GSP concentrations: a. Control; b. 40 µg/ml; c. 50 µg/ml; d. 60 µg/ml; e. 70 µg/ml; f. 80 µg/ml; g. 90 µg/ml; h. 100 µg/ml.

CONCLUSION

The GSP has distinct solubility in DMSO a versatile polar aprotic solvent for many water insoluble drugs. The methodology developed using ethyl acetate fractional precipitation of the extracted GSP uniquely enables the separation and partial purification of the compound. By using conventional acid base precipitation described elsewhere it is not possible to isolate the compound in its original form without eliciting significant chemical modification. Spectral characterizations reveal the protomelanin nature of GSP which forms the basic unit in macro melanin molecules and the ability of the compound to absorb electromagnetic radiation across the spectrum. FTIR analysis indicated the presence of aromatic ring associated with phenolic groups and also the possible presence of amide groups. MTT assay reveals that GSP is highly potent against A549 lung cancer cell lines in *invitro* at viable concentrations to be therapeutic drug. Another significant factor is the close resemblance of the compound to melanin which is a universally biocompatible compound along different genera of organisms, hence possessing the potential for low toxicity. The compound can be further characterized for exact molecular mass and structure which pose is own challenge owing to its sparse solubility in other solvents than DMSO. Further studies focusing on the mechanisms of GSP cytotoxicity in human cancer cell lines are in progress. This study highlights potential strategies or the development of new antitumor compounds with improved therapeutic properties, its utility in combinatorial biosynthesis approaches.

Abbreviations

GSP –Green Sporal Polyketide

EC50 – Effective concentration

MTT assay - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

ISP - International Streptomyces Project

REFERENCES

1. Merzaeva, O. V., & Shirokikh, I. G. Colonization of plant rhizosphere by actinomycetes of different genera. *Microbiology*, 2006; 75(2): 226-230.
2. Ahemad, M., & Kibret, M. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University-Science*, 2014; 26(1): 1-20.

3. Bonaldi, M., Chen, X., Kunova, A., Pizzatti, C., Saracchi, M., & Cortesi, P. Colonization of lettuce rhizosphere and roots by tagged *Streptomyces*. *Frontiers in microbiology*, 2015; 6.
4. Ensign, Jerald C. "Formation, properties, and germination of actinomycete spores." *Annual Reviews in Microbiology*, 1978; 32.1: 185-219.
5. Quirós, L. M., Hardisson, C., & Salas, J. A. Isolation and properties of *Streptomyces* spore membranes. *Journal of bacteriology*, 1986; 165(3): 923-928.
6. Davis, N. K., & Chater, K. F. Spore colour in *Streptomyces coelicolor* A3 (2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. *Molecular microbiology*, 1990; 4(10): 1679-1691.
7. Yu, T. W., Shen, Y., McDaniel, R., Floss, H. G., Khosla, C., Hopwood, D. A., & Moore, B. S. Engineered biosynthesis of novel polyketides from *Streptomyces* spore pigment polyketide synthases. *Journal of the American Chemical Society*, 1998; 120(31): 7749-7759.
8. Gabriella H. Kelemen, Paul Brian, Klas Fla^oRdh, Leony Chamberlin, Keith F. Chater and Mark J. Buttner. Developmental Regulation of Transcription of *whiE*, a Locus Specifying the Polyketide Spore Pigment in *Streptomyces coelicolor* A3 (2). *Journal of Bacteriology*, 1998; 180(9): 2515–2521.
9. Kannan, N. Laboratory Manual in General Microbiology. Second Edition. Panima Publishing Corporation. 2002; ISBN 821-86535-40-5.
10. Holt JA, Krieg NR, Sneath PHA (1994) Bergey's manual of determinative bacteriology. Baltimore, MD.
11. Shirling, E.B. and Gottlieb, D. Methods of Characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology*., 1966; 16(3): 313-340.
12. Keum-Young Jeon, Jin-Hyun Kim. Improvement of fractional precipitation process for pre-purification of paclitaxel. *Process Biochemistry*., 2009; 44: 736–741.
13. Funa, N., Funabashi, M., Ohnishi, Y., & Horinouchi, S. Biosynthesis of hexahydroxyperylenequinone melanin via oxidative aryl coupling by cytochrome P-450 in *Streptomyces griseus*. *Journal of bacteriology*, 2005; 187(23): 8149-8155.
14. Maurya, D. K., Nandakumar, N., & Devasagayam, T. P. A. Anticancer property of gallic acid in A549, a human lung adenocarcinoma cell line, and possible mechanisms. *Journal of clinical biochemistry and nutrition*, 2011; 48(1): 85.
15. Khamna, S., Yokota, A., & Lumyong, S. Actinomycetes isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid

- and siderophore production. *World Journal of Microbiology and Biotechnology*, 2009; 25(4): 649-655.
16. Vijayabharathi, R., Bruheim, P., Andreassen, T., Raja, D. S., Devi, P. B., Sathyabama, S., & Priyadarisini, V. B. Assessment of resistomycin, as an anticancer compound isolated and characterized from *Streptomyces aurantiacus* AAA5. *The Journal of Microbiology*, 2011; 49(6): 920-926.
17. Che, Q., Zhu, T., Keyzers, R. A., Liu, X., Li, J., Gu, Q., & Li, D. Polycyclic hybrid isoprenoids from a reed rhizosphere soil derived *Streptomyces* sp. CHQ-64. *Journal of natural products*, 2013; 76(4): 759-763.
18. Raju, R., Gromyko, O., Fedorenko, V., Luzhetskyy, A., Plaza, A., & Müller, R. Juniperolide A: a new polyketide isolated from a terrestrial actinomycete, *Streptomyces* sp. *Organic letters*, 2012; 14(23): 5860-5863.